AD_____

Award Number: DAMD17-01-1-0372

TITLE: Dendritic Cell-Based Immunotherapy of Breast Cancer: Modulation by CpG DNA

PRINCIPAL INVESTIGATOR: Joseph Baar, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, Pennsylvania 15260

REPORT DATE: September 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2003	Annual (15 Aug 2002 - 14 Aug 2003)

4. TITLE AND SUBTITLE
Dendritic Cell-Based Immunotherapy of Breast Cancer: Modulation by CpG DNA

5. FUNDING NUMBERS
DAMD17-01-1-0372

6. AUTHOR(S)
Joseph Baar, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pittsburgh
Pittsburgh, Pennsylvania 15260

E-Mail: barrj@msx.upmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Breast cancer is the most common non-skin cancer in women, and the American Cancer Society estimates that there will be 203,500 new cases of invasive breast cancer and 40,000 deaths form metastatic breast cancer (MBC) in the U.S. in 2002. Thus, patients with MBC who fail conventional therapies are candidates for clinical trials using novel therapies, including immunotherapy. Dendritic cells (DC) are potent antigen-presenting cells that prime antitumor cytotoxic T lymphosytes against tumor-associated antigens and bacterial DNA oligodeoxynucleotides containing unmethylated CpG sequences (CpG DNA) further augment the immune priming functions of DCs. We hypothesize that CpG DNA-stimulated DCs will prime a more potent anti-tumor immune response than non-stimulated DCs. In year 2 of this proposal, our 2 specific aims are 1) to study the mechanism of antitumor immunity mediated by the vaccination of TS/A mammary tumor-bearing BALB/c mice with CpG DNA-stimulated DCs primed in vitro withnecrotic TS/A cells and 2) to determine optimal conditions for CpG DNA stimulation and tumor priming of human DCs.

14. SUBJECT TERMS
Immunology, dendritic cells, CpG DNA

15. NUMBER OF PAGES
15

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

I. INTRODUCTION

Dendritic cells (DC) are potent antigen-presenting cells that prime antitumor immunity against tumor-associated antigens by cytotoxic T lymphocytes, and bacterial DNA oligodeoxynucleotides containing unmethylated CpG sequences (CpG DNA) can further amplify the immunostimulatory capacity of DCs. In this proposal we hypothesize that CpG DNA-stimulated DCs will prime a more potent anti-tumor immune response than non-stimulated DCs. In Aim 1 of this proposal, we test this hypothesis in a pre-clinical model of breast cancer using an established mammary tumor (TS/A) in syngeneic BALB/c mice. DCs are primed by necrotic tumor cells *in vitro* and then injected subcutaneously with CpG DNA in TS/A tumor-bearing mice. In Aim 2, as a prelude to a future clinical trial, we determine the optimal conditions for tumor priming and CpG DNA stimulation of human DCs. In this aim, we will develop optimal loading conditions of human DCs with tumor lysate derived from the MCF-7 human breast cancer cell line. In Aim 3, based on the pre-clinical data generated in Aims 1 and 2, we plan to undertake a Phase I/II clinical trial of immunization with autologous tumor-primed DCs and dose-escalated CpG DNA in patients with metastatic breast cancer.

II. BODY

Aim 1. To Study The Mechanism Of Antitumor Immunity Mediated By The Vaccination Of TS/A Tumor-Bearing Mice With CpG DNA-Stimulated DCs Primed In Vitro With Necrotic TS/A Cells.

In the first series of experiments, 55 mice were treated in 9 groups - 5 mice/group for treatment groups 1-7, and 10 mice/group for treatments 8 and 9. The 9 groups of BALB/c mice were injected subcutaneously in the left flank with 10^5 TS/A tumor cells and the tumors were allowed to grow until a nodule was palpable. While tumors were growing *in vivo*, DCs were loaded *in vitro* with tumor lysate from TS/A cells (DC^{TL}). Tumor lysates were generated by repeat cycles of freezing and thawing, followed by co-culture with DCs for 24 hours. The 9 groups of mice were then injected on Day 8 (post-tumor inoculation) in the right flank and boosted one week later (Day 15) with either (1) PBS, (2) CpG DNA (5'-TCCATGACGTTCCTGATGCT-3'), (3) non-CpG DNA (5'-TCCATGAGCTTCCTGATGCT-3'), (4) DCs alone, (5) DC + non-CpG DNA, (6) DC + CpG DNA, (7) tumor lysate-loaded DCs (DC^{TL}), (8) DC^{TL} + non-CpG DNA, or (9) DC^{TL} + CpG DNA, respectively. We used ~ $5x10^5$ DCs and 0.1μM DNA in our injections. Tumor size was then measured twice a week in a blinded fashion. However, we did not notice a significant reduction in tumor growth in mice treated with DC^{TL} + CpG DNA when compared to control groups. We therefore repeated these experiments using a larger quantity of CpG DNA in a dose-escalated fashion. Because we were only interested at this point in determining the optimal dose of CpG DNA for tumor rejection, we did a rapid screen using 3 BALB/c mice per group for the following 5 groups: 1) PBS; 2) DC^{TL}; 3) DC^{TL} + 1 nmole CpG; 4) DC^{TL} + 3.3.nmoles CpG; 5) DC^{TL} + 10 nmoles CpG. A depicted in Fig. 1, we saw some minor slowing of tumor growth at the highest concentration of CpG DNA (10 nmoles). We were therefore interested in determining whether we could achieve further growth delay by using even higher doses of CpG DNA. Thus, we repeated the same experiment using 5 groups of 3 BALB/c mice injected with: 1) PBS; 2) DC^{TL}; 3) DC^{TL} + 10 nmoles CpG; 4) DC^{TL} + 33 nmoles CpG; 5) DC^{TL} + 100 nmoles CpG. As seen in Fig. 2, we noted tumor regression at

both the 33 and 100 nmole levels of CpG DNA. However, when the booster injection (Injection 2) was given on Day 13, 1 of the 3 mice in the 33-nmole group and 2 of the 3 mice in the 100-nmole group died within hours of the injections. No mice died in the 10 nmole group.

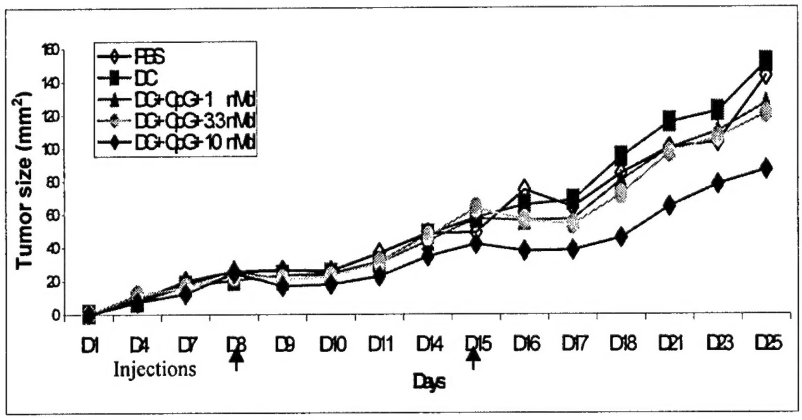


Fig. 1. Therapy of TS/A Bearing BALB/c Mice with DC +/- CpG DNA (1, 3.3, and 10 nmoles)

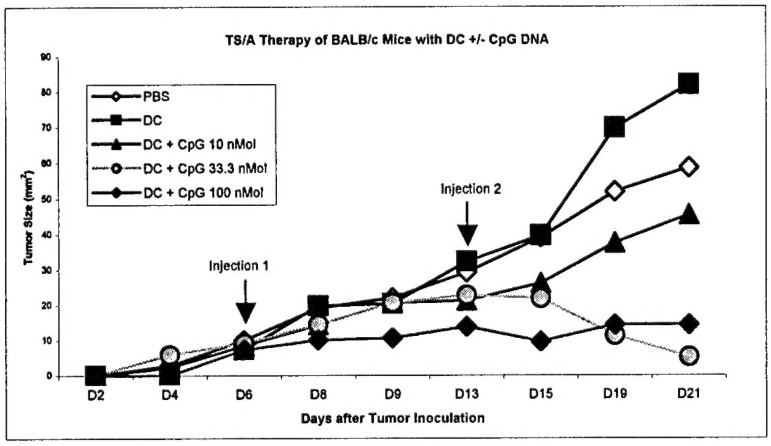


Fig. 2. Therapy of TS/A Bearing BALB/c Mice with DC +/- CpG DNA (10, 33, and 100 nmoles)

Thus, the toxicities were related to the dose of CpG DNA administered in the booster injections. As no necropsies were performed on the dead mice, the reason for this CpG DNA dose-dependent toxicity is unclear, but may be related to the generation of a "cytokine storm" *in vivo* by the booster injection. To wit, we have demonstrated that CpG DNA can induce the production of large amounts of IL-12, IFN-γ, TNF-α and NO by murine DCs *in vitro*, and the production of large quantities of such cytokines *in vivo* might generate significant toxicities, including death. Therefore, since 33 nmoles of CpG DNA mediate tumor rejection, we want to determine a booster dose of CpG DNA that is less toxic to mice and yet is effective enough to mediate tumor regression. In order to determine this CpG DNA booster dose, we will repeat the above experiment in 7 groups of 3 BALB/c mice by giving 33 nmoles of CpG DNA with the first injection of DCs, followed by a dose-escalated amount of CpG DNA in the booster injections. There will also be a CpG DNA-alone control group where mice will receive 33 nmoles DNA in both injections. The groups are:

Group 1. PBS
Group 2. DC^{TL}
Group 3. CpG: 33 nmoles (injection 1); 33 nmoles (booster injection)

Group 4. DC^{TL} + 33 nmoles CpG (injection 1); 1 nmole CpG DNA (booster injection)
Group 5. DC^{TL} + 33 nmoles CpG (injection 1); 5.5 nmoles CpG DNA (booster injection)
Group 6. DC^{TL} + 33 nmoles CpG (injection 1); 11 nmoles CpG DNA (booster injection)
Group 7. DC^{TL} + 33 nmoles CpG (injection 1); 22 nmoles CpG DNA (booster injection)
Group 8. DC^{TL} + 33 nmoles CpG (injection 1); 33 nmoles CpG DNA (booster injection)

Once the optimal dosing scheme for CpG DNA is determined, we plan to repeat the formal assessment of this therapeutic approach by repeating the experiments with the original 9 groups of mice intended in this Task, as detailed at the beginning of this section.

Aim 2. To Determine Optimal Conditions For CpG DNA Stimulation And Tumor Priming Of Human DCs.

Many DC-based human clinical therapy protocols utilize many cytokines (e.g. hGM-CSF and hIL-4) and maturation factors (e.g. LPS, IL-1, TNF-α, PGE2, poly I:C) to direct peripheral blood monocytes to differentiate into monocyte-derived DC (mDC). This process is cumbersome, expensive and generally requires 7 to 8 days of *in-vitro* culture to generate functional DCs. The endpoint of this grant proposal is to develop a simpler and more efficient DC clinical protocol to treat patients with metastatic breast cancer where CpG DNA will be used as the single, primary stimulus without using any other maturation factors to drive human peripheral blood DC precursors into mature DCs.

A subset of human DCs responsive to CpG DNA has been characterized by Krug *et al* (1). These plasmacytoid DC (pDC, CD123+) express the Toll-like receptor-9 (TLR9) which is involved in the recognition of CpG motifs. The blood dendritic cell antigen-4 (BDCA-4) is a unique pDC marker that allows for the isolation of these pDCs by elution from a BDCA-4 selection column. The authors demonstrated that a CpG DNA (5'-TCGTCGTTTTGTC GTTTTGTCGTT-3') supported the survival, activation (CD80, CD86, CD40, MHC class II), chemokine production (IL-8, IP-10) and maturation (CD83) of BDCA-4 selected pDC which were grown in culture medium containing hGM-CSF and hIL-3. In another study, Gursel *et al* (2) used another CpG DNA (5'-GTGCATCGATGCAGGGGGG-3') to stimulate human peripheral blood monocytes to mature into functionally active DC over 2-4 days. The transition from monocyte to DC was characterized by the up-regulation of CD83, CD86, CD80, CD40 and the down-regulation of CD14 in serum- and cytokine-free medium (XVIVO) containing only CpG DNA as the maturation factor. The differentiation of these monocytic DC (mDC) precursors to functional DCs was mediated by pDC present at low frequency in the peripheral blood sample, which responded to the CpG DNA by secreting IFN-alpha which, in turn, induced the maturation of the mDCs. The 2 major advantages of this latter approach compared to the former is that, first, no column selection procedure is required to isolate DCs, thereby significantly increasing the yield of mature DCs and, second, no cytokines (GM-CSF or IL-4 or IL-3) are required in the growth medium to sustain DC growth and maturation. We were therefore interested in doing a head-to-head comparison of the ability of these 2 CpG DNAs to stimulate human DCs.

OBJECTIVES

The objectives of this task were to:

1. Test the 2 above-mentioned CpG DNAs on both pDC and mDC and compare their effect on DC maturation (e.g. upregulation of MHC Class II, CD40, CD86, etc).
2. Test MCF-7 tumor lysate uptake by DCs after CpG stimulation and determine whether DC maturation markers are altered by tumor lysate loading.
3. Use autologous DC-primed T cells in an *in-vitro* cytotoxicity assay against MCF-7.

MATERIALS AND METHODS

CpG DNAs. 1: 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3' (used for pDC maturation or as indicated); 2: 5'-GGTGCATCGATGCAGGGGGG-3' (used for mDC maturation or as indicated).

Generation of pDC. Peripheral blood mononuclear cells (PBMC) were isolated form health human donors by Ficoll gradient separation. PDC were selected from PBMC by anti-BDCA-4 conjugated magnetic beads. pDC eluted from separation column were cultured in the presence of hGM-CSF and hIL-3 for 1 day (Fig. 3).

Generation of mDC. PBMC were separated by Percoll gradients into lymphocyte and monocyte sections. Monocytes were cultured in XVIVO® medium in the presence of CpG DNA but without any other cytokines or maturation factors. The rationale for the use of unselected mDCs is that these aliquots contain both pDC and mDCs. The addition of CpG DNA to the growth medium stimulates the pDCs to produce IFN-α which, in turn, stimulate the maturation of the mDCs in the culture (Fig. 3).

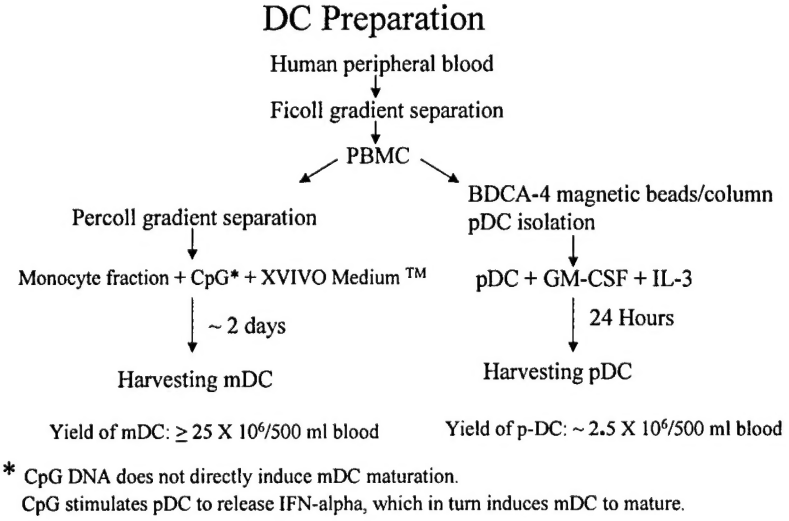


Fig. 3. Preparation of pDC and mDC from human peripheral blood

DC Surface Markers. Markers were detected through fluorescent-conjugated antibody to CD80, CD86, CD83, CD40, class II and etc by flow cytometer.

Tumor Antigen Loading. The human breast cancer cell line (MCF-7) lysate was prepared through repeated freeze and thaw cycles and was added overnight to the DC preparation in a 1:1 ratio of tumor cells to DCs. The next day, the loaded DCs were washed and labeled for FACS analysis.

Tumor Labeling. To monitor tumor uptake by DC, tumor cells were stained with DiI before loading onto DC.

Cytotoxicity Assays. Monocytes isolated from PBMC were grown for 1 day in XVIVO medium containing CpG DNA. The breast cancer cell line MCF-7 was lysed by repeated freezing and thawing. Tumor lysate was added to DC at the ratio DC: Tumor = 1 :1 for 1 day. After harvesting the tumor-pulsed DCs, they were used to prime autologous T cells (PBMC) at the ratio T:DC = 10:1. Il-2 (20 u/ml) was added to the medium on Day 5. T cells were restimulated every week with the CpG-treated DC. CTL were harvested after 3 stimulations and used as effectors against MCF-7 in an LDH cytotoxicity assay (Promega).

RESULTS

1. Test CpG DNA On Both pDC And mDC And Compare Their Responses By DC Markers.

We observed that both CpG DNAs were equally effective in upregulating both pDC- and mDC-associated cell-surface markers such as MHC class II, CD40, CD80 and CD83. Maximum upregulation of these cell-surface markers was usually seen after 48 hours of DC culture (Figs. 4a and 4b).

a) CpG DNA-1 Upregulates pDC Marker Expression

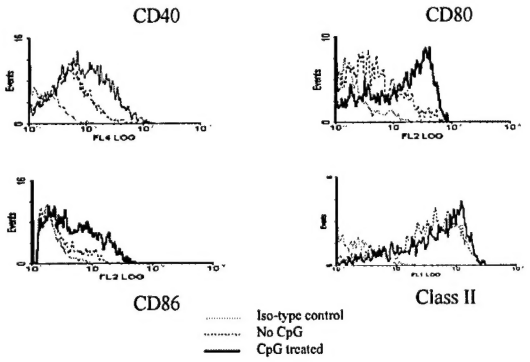
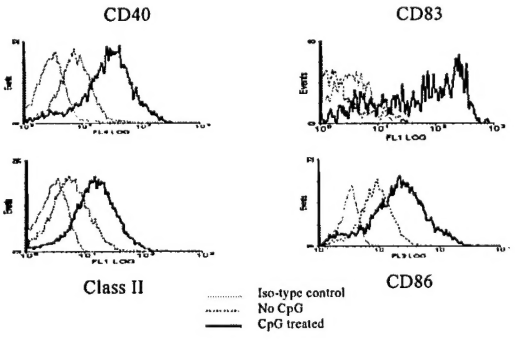


Fig 4. Upregulation of Maturation Markers by CpG DNA in a) pDC and b) mDC

b) CpG DNA-2 Upregulates mDC Marker Expression



Since CpG DNA-1 is commercially available and CpG DNA-2 is not, and since CpG DNA-1 has been tested for its ability to induce the maturation of pDC but not mDC in bulk peripheral blood, we tested whether CpG DNA-1 could induce the maturation of mDC. Thus, peripheral blood monocytes were grown in XVIVO medium containing 3 μM of CpG DNA-1. As shown in Fig. 5, this CpG DNA was equally effective in stimulating the maturation of both pDCs and mDCs.

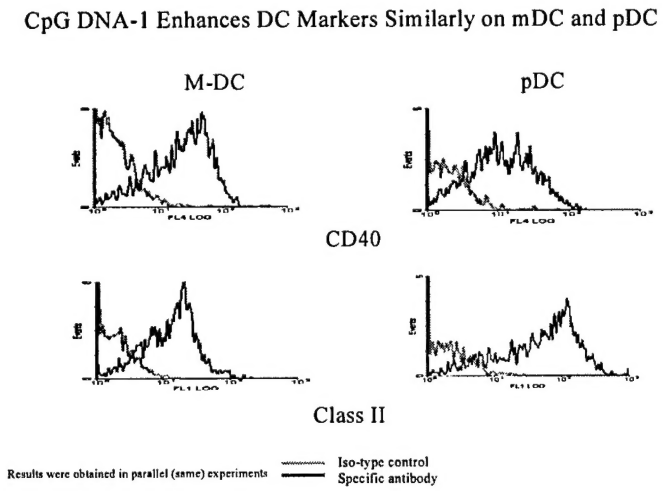


Fig. 5. CpG DNA-1 Is Equally Effective In Inducing The Maturation Of pDCs and mDCs

Therefore, we elected to use CpG DNA-1 in all of our subsequent experiments, as detailed below.

2. *Test MCF-7 Tumor Lysate Uptake By DCs After CpG Stimulation And Determine Whether DC Maturation Markers Are Altered By Tumor Lysate Loading.*

We observed that mDCs were efficient in taking up lysate from the MCF-7 human breast cancer cell line and that cell-surface DC marker expression was not reduced after tumor lysate loading (Fig. 6).

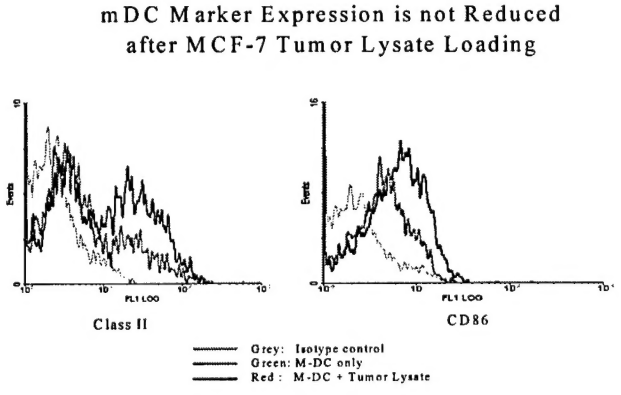


Fig. 6. mDC Marker Expression is not Reduced after MCF-7 Tumor Lysate Loading

3. *Use Autologous DC-Primed T Cells In An In-Vitro Cytotoxicity Assay Against MCF-7.*

When autologous CTLs were primed by mDCs loaded with MCF-7 tumor lysate, we observed a dose-dependent killing of MCF-7 (Fig. 7)

CTL: Killing of MCF-7 Human Breast Cancer Cells by T Cells Primed with CpG Treated DC Pulsed with MCF-7 Tumor Lysate

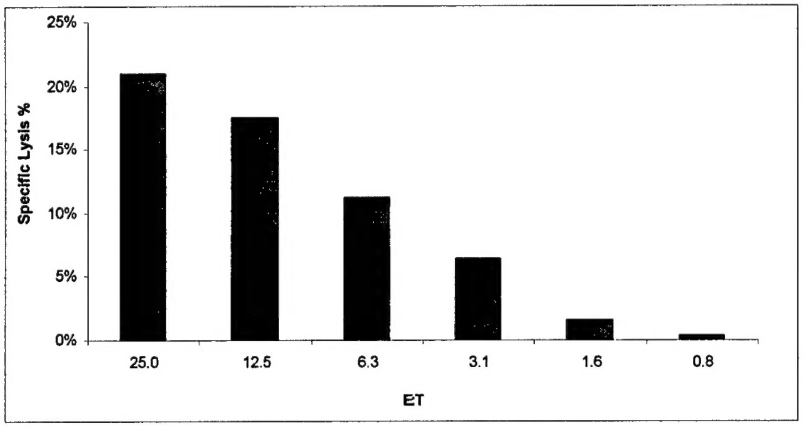


Fig. 7. Killing of MCF-7 Human Breast Cancer Cells by T Cells Primed with CpG DNA Treated DC Pulsed with MCF-7 Tumor Lysate

Aim 3. To Undertake A Phase I/II Clinical Trial Of Immunization With Autologous Tumor-Primed DCs And Dose-Escalated CpG DNA In Patients With Metastatic Breast Cancer.

Once we have completed the studies detailed in Aims 1 and 2, we will undertake a clinical trial of therapy of women with MBC using tumor-loaded DCs and CpG DNA. Although the final details of the protocol will depend on the results obtained in Aims 1 and 2, the general outline of the protocol will be as follows:

This will be a Phase I/II clinical trial of immunization with autologous tumor-primed dendritic cells (DC) and dose-escalated CpG DNA in patients with metastatic breast cancer (MBC). The Phase I portion of the clinical trial will determine a starting dose of CpG DNA for the Phase II trial, to be followed by the Phase II trial which will evaluate the efficacy of the treatment under consideration.

1. Pre-Treatment Plan

A. Patient Selection

- Patients must have biopsy-proven MBC.
- Patients must have sufficient resectable tumor volume (at least 10^9 tumor cells, or a 1 cm^3 nodule) for vaccine preparation.
- Patients must have measurable MBC after resection of the tumor nodule.
- Patients must have reactivity to at least one of four PCI skin tests (see below).

- Patients must have fully recovered from surgery, and must not have received any chemotherapy, radiotherapy, hormonal therapy, or immunotherapy within 4 weeks preceding vaccination.
- Patients with treated brain metastases are eligible.
- Patients requiring therapy with steroids are not eligible.
- Patients must have an expected survival of at least three months.
- Patients must have an ECOG Performance Status (PS) of ≤ 1 (Karnofsky PS ≥ 80).
- Patients must have the following initial and subsequent pretreatment laboratory parameters:

Granulocytes:	>2,000/mm^3
Lymphocytes:	>700/mm^3
Platelets:	>100,000/mm^3
Serum Creatinine:	< 2.0 mg/100 ml
Serum Bilirubin:	< 2.0 mg/100 ml

- Patients must be able to give written informed consent.
- Patients must not be pregnant or lactating.

B. Investigations

All patients will undergo the following investigations ≤ 4 weeks prior to DC vaccination:

- Bloodwork: CBC, differential, platelets, electrolytes, BUN, creatinine, AST, ALT, alkaline phosphatase, total bilirubin, albumin, Ca++.
- Radiology:
  - ✧ CT of the chest and abdomen
  - ✧ Total body bone scan
  - ✧ If clinically indicated, a MRI or CT scan of the brain
- PCI skin test (see below)

C. PCI Skin Test

- Following evaluation and meeting the eligibility criteria noted above, the multitest PCI skin test with antigens for cellular hypersensitivity will be applied in initial patient screening (≤ 4 weeks prior to DC vaccination).
- Each test contains a disposable applicator which is preloaded with 1 of 4 antigens to be delivered intradermally, including tetanus, tuberculin, Candida, mumps, and a normal saline solution control.
- Induration will be measured at 24 and 48 hours.

2. Treatment Plan

A. DC Vaccine Preparation

- The autologous tumor lysate-pulsed DCs will be prepared in the Immune Monitoring and Cellular Products Laboratory (IMCPL) of the University of Pittsburgh Cancer Institute.

- DC precursor cells obtained from patients' buffy coat product will be cultured for 1 day in serum-free X-VIVO® medium containing 3 μM CpG DNA (5'-TCGTCGTTTTGTCGTT TTGTCGTT-3') to generate DCs.
- Resected tumor specimens will undergo 5 freeze/thaw cycles to induce cell lysis.
- DCs will be then be pulsed overnight with tumor lysate or hepatitis B surface antigen @ 37°C and washed next day 3 times with sterile saline.
- The DC vaccine will then be split into 3 aliquots:
  - ✧ ⅓ of the DC vaccine will be used for the first course of DC vaccination.
  - ✧ ⅓ of the DC vaccine will be frozen and then thawed and cultured for 1 day prior to the 2nd DC vaccination.
  - ✧ ⅓ of the DC vaccine will be frozen and used for *in-vitro* assays as detailed below.

B. DC Vaccine Administration

- Tumor lysate- or hepatitis B surface antigen-pulsed DCs will be counted and combined into a single syringe, respectively, with the scheduled dose of CpG DNA for intradermal injection.
- The DC vaccine will be administered intradermally at Weeks 1 and 2.
- It is expected that at least 10^6 DCs will be administered with each cycle of vaccination.
- All patients will be treated in the NIH-funded General Clinical Research Center at the University of Pittsburgh Cancer Institute.

3. Treatment Endpoints

A. Clinical Endpoints

Phase I Trial. In Phase I, a predetermined number of groups of patients with measurable MBC will receive 2 intradermal injections (2 cycles) given 1 week apart consisting of at least 10^6 tumor-loaded DCs admixed with an escalating dose of CpG DNA. The safety of individual dose tiers will be evaluated by treating 2 patients at each of the lower tiers and observing patients for toxicity for 2 weeks before treating a new patient at the next higher dose. Six patients will be treated at the maximal dose of CpG DNA to more fully evaluate the toxicity profile at that level. If a grade III/IV (NCI common criteria) toxicity is observed the dose escalation scheme will immediately switch to the conventional phase I design for finding MTD (3 to 6 patients per tier). Immunologic monitoring as detailed below will also be undertaken

Phase II Trial. The Phase II trial will start at the CpG dose determined in Phase I. The Phase II portion of the trial will be carried out as detailed in *Statistics* below. The endpoints of this phase of the clinical trial will be 1) to measure tumor response to the DC^{TL} + CpG DNA vaccine, and 2) to define the immunologic response to autologous tumor induced in patients who have received the DC^{TL} + CpG DNA vaccine as detailed below. Objective evidence of tumor regression will be measured using the RECIST criteria.

B. Immunologic Endpoints

ELISPOT Assay. Peripheral blood (60 ml) will be drawn one week before the first vaccination and one week after the second vaccination. Peripheral blood lymphocytes (PBL) (20 x 10^6) will be incubated in tissue culture tubes in 4 ml RPMI-1640, 10% heat-inactivated human AB serum, antibiotics, and 50 IU/ml rhIL-2. After 6 days, T cells will be evaluated in ELISPOT assays. Pre- and post-vaccination PBL (at least 10^7) will be separated into CD4+ and CD8+ fractions by selection on immunobeads. The enriched fractions will be counted and tested by flow cytometry to determine the percent of CD8+ and CD4+ T cells in each. The enriched fractions will be tested in 20h ELISPOT assays performed in triplicate wells, each containing 50,000 cells/well. The ratio of T cells to DC will be 10:1. The assays will be formatted as follows:

- ✧ CD8+T cells alone.
- ✧ CD4+T cells alone.
- ✧ CD8+T cells + irradiated tumor alone.
- ✧ CD4+T cells + irradiated tumor alone.
- ✧ CD8+T cells + unprimed DC.
- ✧ CD4+T cells + unprimed DC.
- ✧ CD8+T cells + tumor-primed DC.
- ✧ CD4+T cells + tumor-primed DC.
- ✧ CD8+T cells + Hepatitis B sAg.
- ✧ CD4+T cells + Hepatitis B sAg.
- ✧ CD8+T cells + DC + Hepatitis B sAg.
- ✧ CD4+T cells + DC + Hepatitis B sAg.

After incubation, the plates will be developed, and the numbers of spots in experimental and control wells will be counted, using a Zeiss image analysis system. The ELISPOT assay results will be expressed as the difference between the mean number of spots in the experimental wells - mean number of spots in the control wells, or as the frequency of IFN-γ-secreting cells in total cells plated per well. The final results will be expressed as the frequency of CD4+ and CD8+ T cells responding to cryptic tumor antigens in the population tested. The expectation is that patients responding to the DC vaccination will demonstrate a significant difference in the frequency of responding CD4+ and CD8+ T cells before and after DC vaccination

4. Statistics

The Phase I clinical trial will determine a starting dose of CpG DNA for a Phase II trial, followed by a Phase II trial to evaluate the efficacy of the treatment under consideration. In the Phase I portion, the dose of CpG DNA will be escalated in tiers to be predetermined according to the pre-clinical data completed in Aims 1 and 2. The safety of the dose tier schedule will be evaluated by treating 2 patients at each of the lower tiers and observing patients for toxicity for 2 weeks before treating a new patient at the next higher dose. Six patients will be treated at the highest dose of CpG DNA to more fully evaluate the toxicity profile at that level. If a grade III/IV (NCI Common Toxicity Criteria) toxicity is observed the dose escalation scheme will immediately switch to the conventional phase I design for finding MTD (3 to 6 patients per tier). The Phase II trial will then commence at the starting dose determined in Phase I. The sample size

will be based upon the two stage design of Simon which allows a trial to be stopped at the end of the first of two stages if the therapy under study shows a poor response rate. Immunologic response endpoints will be summarized with point estimates and confidence intervals. When pre-treatment measurement are available, vaccine-mediated effects will be examined via either a signed rank test for continuous data or McNemar's test for binary data. The immunologic measurement to be evaluated includes pre- to post-vaccination T cell reactivity as measured by the ELISPOT assay. Since this is a vaccine trial with a variable number of tumor-pulsed dendritic cells available for the vaccine, immunogenic responses will be compared to the final number of delivered DCs in the vaccine.

III. KEY RESEARCH ACCOMPLISHMENTS

Aim 1. We have demonstrated that we can induce the regression of a TS/A tumor in BALB/c mice using tumor-lysate loaded DCs and CpG DNA dosed at both 33 and 100 nmoles. However, significant toxicity was encountered when mice that had been primed with DC + 100 nmoles CpG DNA were boosted with a second dose of 100 nmoles of CpG DNA. There was less toxicity at the 33 nmole dose with equal efficacy. We will therefore be repeating experiments where the initial dose of CpG DNA will still be 33 nmoles but the booster dose of CpG DNA will be carefully escalated to mediate tumor regression with no toxicity. We are confident that this line of investigation will be successful in identifying the optimal dosing schema of CpG DNA in order to induce the regression of established TS/A tumors in our BALB/c mice.

Aim 2. We have demonstrated that activated mDCs can be generated from growth in serum-free medium containing CpG DNA-1 and the resultant DCs are efficient in taking up tumor lysate with no downregulation of maturation markers on mDCs. We have also demonstrated that these mDCs are efficient at priming an autologous T cell cytotoxic response against MCF-7. To score specifically for a CTL-mediated cytotoxic response, we will complete the work in this Aim with a cytotoxicity assay using mDC and CTL from HLA-A2(+) donors against MCF-7 which is HLA-A2(+). CTL-specificity will be scored by observing a decrease in MCF-7 killing in the presence of an anti-HLA-A2 antibody.

IV. REPORTABLE OUTCOMES.

We have submitted our human data as an abstract to the Meeting of the International Society of Biological Therapy (10/03) and we plan to submit our murine data as an abstract to the Meeting of the American Association of Cancer Research (3/04). Once we complete both of these studies, the manuscripts will be submitted for publication to such leading journals as Cancer Research, Journal of Immunology, Journal of Experimental Medicine, or other.

V. CONCLUSIONS

In conclusion, we have compelling pre-clinical data to suggest that a DC-based approach to *in-vivo* cancer therapy is augmented by the use of CpG DNA (Aim 1) and that human autologous cytotoxic T cells can be primed by tumor lysate-loaded mDCs to mediate the killing of a human breast cancer cell line (Aim 2). The 2 major advantages of our adopted approach for the generation of human mDCs is that (1) it obviates the need for expensive cytokine growth factors and (2) functional DCs can be generated within 48 hours for cancer therapy. It is hoped that these

studies will provide a novel and non-toxic immunotherapeutic approach to the treatment of women with metastatic breast cancer (Aim 3).

VI. REFERENCES

1. Krug A, Towarowski A, Britsch S, Rothenfusser S, Hornung V, Bals R, Giese T, Engelmann H, Endres S, Krieg AM, Hartmann G. 2001. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. Eur. J. Immunol. 31:3026-3037.
2. Gursel M, Verthelyi D, Klinman D.M. 2002. CpG oligodeoxynucleotides induce human monocytes to mature into functional dendritic cells Eur. J. Immunol. 32: 2617-2622.